PHYLOGENETIC RELATIONSHIPS OF TWO ENDEMIC GENERA FROM EAST ASIA: *TRICHOCOLEOPSIS* AND *NEOTRICHOCOLEA* (HEPATICAE)[1]

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ABSTRACT

Trichocoleopsis S. Okamura and *Neotrichocolea* S. Hatt. are two water saccate liverwort genera endemic to East Asia whose systematic positions have been controversial. To address their phylogenetic positions and relationships within liverworts, two data sets were analyzed using maximum parsimony and maximum likelihood methods: (1) four genes (*psbA*, *rps4*, 26S, and *nad5*) of 57 taxa, and (2) nine genes (chloroplast small subunit [SSU], large subunit [LSU], *atpB*, *rbcL*, *psbA*, and *rps4*; nuclear ribosomal 18S and 26S; mitochondrial *nad5*) of 24 taxa. All of our analyses suggested the sister relationship between *Trichocoleopsis* and *Neotrichocolea*, which is also supported by morphological characters, such as a lobed leaf with ciliate margins, water sacs, coelocaules, and interior capsule wall cells with thin walls. Traditional classifications usually considered *Trichocoleopsis* and *Neotrichocolea* as members of Lepidolaenaceae or Trichocoleaceae, whereas our results strongly suggested that the *Trichocoleopsis–Neotrichocolea* clade allied with Ptilidiaceae, which is supported by the similar ciliate and lobed leaves. However, considering many differences between them, such as in the perianth, sporeling type, water sacs, and rhizoids, we support a family rank for the Neotrichocoleaceae, containing *Trichocoleopsis* and *Neotrichocolea*, as previously suggested by Inoue in 1974.

Key words: East Asia, endemic, multiple genes, *Neotrichocolea*, Neotrichocoleaceae, phylogeny, *Trichocoleopsis*.

Trichocoleopsis S. Okamura and *Neotrichocolea* S. Hatt. are two liverwort genera from East Asia that are considered to be related based on the shared characteristic of bearing water sacs on their leaves. *Trichocoleopsis* contains two species, *T. sacculata* (Mitt.) S. Okamura and *T. tsinlingensis* P. C. Chen ex M. X. Zhang, and the latter is only distinguished from the former by its entire or only 1- to 3-ciliate lobe margins (Zhang, 1982). *Neotrichocolea* is a monotypic genus with *N. bisseti* (Mitt.) S. Hatt. The two genera are restricted to moist forests in China, Japan, Korea, Burma, and the Russian Far East. As habitat destruction increases, all three species of both genera are seriously threatened and on the verge of extinction (Wu et al., 1997; Cao et al., 2006). An updated distribution map of these species is shown in Figure 1. This map is based on our own consultations of specimens as well as other published research (see Table 1).

In 1891, Mitten published two species from Japan, *Blepharozia sacculata* Mitt. and *Mastigophora bisseti* Mitt; the former is the basionym of *Trichocoleopsis sacculata* and the latter is the basionym of *Neotrichocolea bisseti*. Thereafter, the two species were transferred to *Ptilidium* Nees (Stephani, 1897; Evans, 1905) before Okamura (1911) and Hattori (1947) established the monotypic genera *Trichocoleopsis* and *Neotrichocolea*, respectively. In addition, Horikawa (1934) suggested that *N. bisseti* be placed in the genus *Trichocoleopsis*.

Since their establishment, the systematic positions of these two genera have been controversial. *Trichocoleopsis* has been placed in Ptilidiaceae (Evans, 1939; Hattori, 1947; Mizutani & Hattori, 1969), Trichocoleaceae (Nakai, 1943; Müller, 1951–1958; Schuster, 1957; Mizutani, 1972), or Lepidolaenaceae (Schuster, 1966); meanwhile, *Neotrichocolea* has been recognized as a member of Ptilidiaceae (Hattori, 1947;

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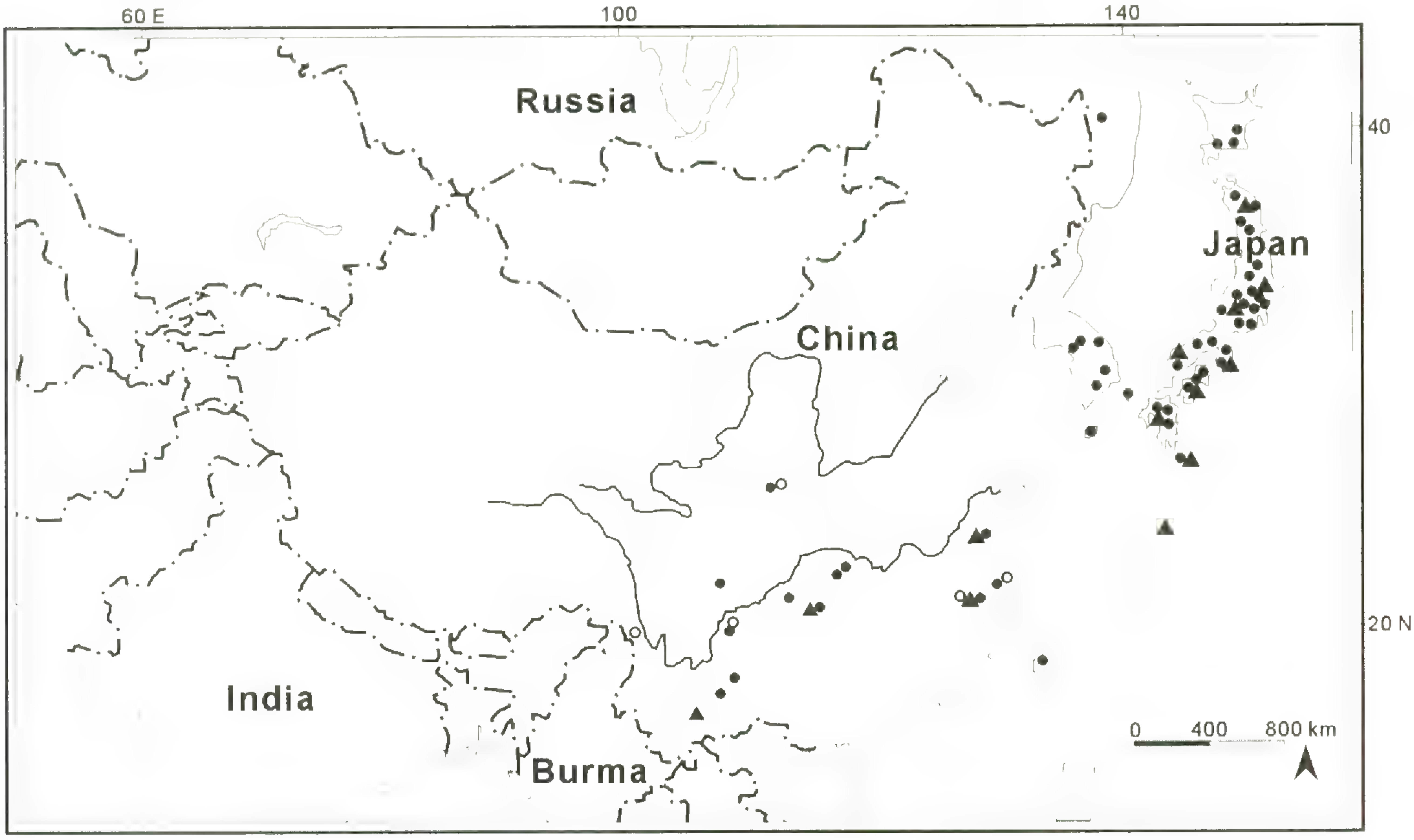

Figure 1. Distribution map of *Trichocoleopsis* and *Neotrichocolea*. Filled circle = *T. sacculata*, open circle = *T. tsinlingensis*, filled triangle = *N. bisseti*.

Table 1. Specimen vouchers for geographic distributions of *Neotrichocolea* and *Trichocoleopsis* in eastern Asia (Fig. 1). Mapped distributions in China and adjacent regions are supported by the specimen vouchers or inferred from the literature (Gao & Cao, 1988, 2000; Hong, 1966 [Korea]; Inoue, 1974 [Japan]; Konstantinova et al., 1992 [Russia]; Koponen et al., 2004; Wu, 1992; Zhang, 1982). Asterisks mark the two specimen vouchers for the new chloroplast and nuclear ribosomal DNA sequences reported in this paper. IFSBH = Herbarium, Kunming Institute of Botany, Chinese Academy of Sciences.

Locality	Collection	Herbarium/ Literature
***Neotrichocolea bisseti* (Mitt.) S. Hatt.**		
Guang mingding, Mt. Huang, Anhui Province, China	*Chen et al. 6900*	PE
Qing liangtai, Mt. Huang, Anhui Province, China	*Chen et al. 7081*	PE
Qing liangtai, Mt. Huang, Anhui Province, China	*Chen et al. 7128*	PE
Shi xinfeng, Mt. Huang, Anhui Province, China	*Chen et al. 7161*	PE
Shi xinfeng, Mt. Huang, Anhui Province, China	*Chen et al. 7104*	PE
Shi xinfeng, Mt. Huang, Anhui Province, China	*Chen et al. 7171*	PE
Shi xinfeng, Mt. Huang, Anhui Province, China	*Chen et al. 7172*	PE
Xi haimen, Mt. Huang, Anhui Province, China	*Chen et al. 7038*	PE
Xi haimen, Mt. Huang, Anhui Province, China (alt. 1600 m)	*Chen et al. 7003*	PE
Lian huafeng, Mt. Huang, Anhui Province, China	*Chen et al. 6939*	PE
Qing liangtai-Er daowan, Mt. Huang, Anhui Province, China	*Chen et al. 7188*	PE
Se-Tse-ling, Mt. Huang, Anhui Province, China (alt. 1800 m)	*Ku, C. M. s.n.*	PE
Bei hai, Mt. Huang, Anhui Province, China (alt. 1600 m)	*Gao, C. H. 21280*	SHM
Bei hai, Mt. Huang, Anhui Province, China (alt. 1600 m)	*Hu, R. L. & Gao, C. H. 8679*	HSNU
Song yanan, Mt. Huang, Anhui Province, China (alt. 1300 m)	*Hu, R. L. & Gao, C. H. 8737*	HSNU
Xi haimen, Mt. Huang, Anhui Province, China	*Chen et al. 9371*	HSNU
Huang gang, Mt. Wuyi, Fujian Province, China	*Gao, C. H. 25077*	SHM
Yuan jiang, Yunnan Province, China	—	Gao & Cao, 2000
*Mt. Fanjing, Guizhou Province, China	*He, L. 040522*	PE
***Trichocoleopsis sacculata* (Mitt.) S. Okamura**		
Shi zilin, Mt. Huang, Anhui Province, China (alt. 1800 m)	*Chen et al. 6399*	PE
Shi zilin, Mt. Huang, Anhui Province, China	*Chen et al. 6384*	PE

Table 1. Continued.

Locality	Collection	Herbarium/ Literature
Shi zilin-Xihai, Mt. Huang, Anhui Province, China	*Chen et al. 6497*	PE
Xi haimen, Mt. Huang, Anhui Province, China	*Chen et al. 6997*	PE
Qing liangtai, Mt. Huang, Anhui Province, China (alt. 1500 m)	*Chen et al. 7172*	PE
Qing liangtai, Mt. Huang, Anhui Province, China	*Chen et al. 7179*	PE
Shi zilin-Yun gusi, Mt. Huang, Anhui Province, China	*Chen et al. 7428*	PE
Feng huangsi, Mt. Jinfo, Sichuan Province, China	*Chen et al. 1632*	PE
San guandian, Mt. Taibai, Shanxi Province, China (alt. 3600 m)	*Li X. J. 705*	PE
Ba xiantai, Mt. Taibai, Shanxi Province, China (alt. 4000 m)	*Li X. J. 718*	PE
*Qing liangtai, Mt. Huang, Anhui Province, China	*Liu Y. 4170*	PE
Qing liangtai, Mt. Huang, Anhui Province, China	*Li D. K. 02274*	SHM
Qing liangtai, Mt. Huang, Anhui Province, China	*Zheng W. F. 14307*	HSNU
Qing liangtai, Mt. Huang, Anhui Province, China (alt. 1600 m)	*Hu, R. L. & Gao, C. H. 8632*	HSNU
Mt. E mei, Sichuan Province, China	*Gao Q. 19374, 19485*	IFSBH
Mt. E mei, Sichuan Province, China	*Gao C. H. 15808, 15946*	Gao & Cao, 1988
Yang yuping, Mt. Jinfo, Chongqing, China (alt. 1400 m)	*Cao T. & Li Q. 41495*	IFSBH
Po niangyan, Daguan, Yunnan Province, China	*Li X. J. 4320*	KUN
Yi liang, Yunnan Province, China	–	Gao & Cao, 2000
Qu jing, Yunnan Province, China	–	Gao & Cao, 2000
Mt. Wuyi, Fujian Province, China	*Gao C. H. 25099*	Gao & Cao, 1988
Bai shanzu Natural Reserve, Zhejiang Province, China (alt. 1010–1600 m)	*Zhu R. L. 90402, 90413, 90554, 90564, 90839, 90883*	HSNU
Mt. Jiu long, Zhejiang Province, China (alt. 1300 m)	*Liu Z. L. 3686*	HSNU
Mt. Xitianmu, Zhejiang Province, China (alt. 1300 m)	*Wang Y. F. 00405*	HSNU
Mt. Fanjing, Guizhou Province, China	*He L. s.n.*	GACP
Mt. Ba dagong, Hunan Province, China	*Koponen 48115, 48168*	Koponen et al., 2004
Mt. Huping, Hunan Province, China	*Koponen 52188*	Koponen et al., 2004
Tai dong, Taiwan Province, China	–	Wu, 1992
***Trichocoleopsis tsinlingensis* P. C. Chen ex M. X. Zhang**		
Mt. Taibai, Shanxi Province, China	*Wei Z. P. 5538*	KUN
Fang yangsi-Ping ansi, Mt. Taibai, Shanxi Province, China (alt. 2800–3000 m)	*Wei Z. P. 5554 (b), 5564 (b)*	Zhang, 1982
De qin, Yunnan Province, China	–	Gao & Cao, 2000
Da guan, Yunnan Province, China	*Li X. J. 4265*	KUN
Mt. Wuyi, Fujian Province, China	*Li D. K. 11604*	SHM
Mt. Wuyi, Fujian Province, China	*Li D. K. & Gao C. H. 11614, 14079, 14141*	SHM
Bai shanzu Natural Reserve, Zhejiang Province, China (alt. 1600 m)	*Zhu R. L. 90529*	HSNU

Müller, 1951–1958; Schuster, 1966) or Trichocoleaceae (Mizutani & Hattori, 1969). Schuster (1972) proposed a subfamily rank, Trichocoleopsidoideae, to include *Trichocoleopsis* under the family Lepidolaenaceae, and later another subfamily, Neotrichocoleoideae, to include *Neotrichocolea* (Schuster, 1979). Grolle (1983) followed these treatments, but because the name of subfamily Trichocoleopsidoideae is illegitimate in nomenclature, he used Trichocoleopsoideae instead. Furuki and Mizutani (1994) placed the two genera in Lepidolaenaceae and kept *Trichocoleopsis* in subfamily Trichocoleopsoideae, but they moved *Neotrichocolea* to subfamily Lepidolaenoideae.

Based on differences in capsule wall anatomy and other structures between the *Trichocoleopsis–Neotrichocolea* group and Lepidolaenaceae, Inoue (1974) proposed a new family, consisting of the two genera, called Neotrichocoleaceae, which was accepted by Schuster (1984). The family Neotrichocoleaceae was also recognized in the recent liverwort classification systems (Crandall-Stotler & Stotler, 2000; Frey & Stech, 2005; He-Nygrén et al., 2006), but the circumscription of the family has been disputed. Frey and Stech (2005) and He-Nygrén et al. (2006) did not list the members of Neotrichocoleaceae, whereas Crandall-Stotler and Stotler (2000) suggested that the family should only include *Neotrichocolea*, and *Trichocoleopsis* should be transferred back to Lepidolaenaceae.

Molecular data ranging from a single gene to multiple genes have been used to resolve the relationships of liverworts (e.g., Capesius & Bopp, 1997; Lewis et al., 1997; Stech et al., 2000; Wheeler, 2000; Boisselier-Dubayle et al., 2002; Davis, 2004; Forrest & Crandall-Stotler, 2004, 2005; He-Nygrén et al., 2004, 2006; Crandall-Stotler et al., 2005; Heinrichs et al., 2005; Forrest et al., 2006; Hentschel et al., 2006). These studies represent important progress in understanding the phylogenetic relationships of liverworts at different levels. These studies also provide a useful instrument for resolving the systematic positions of previously unsampled taxa whose placements are controversial or uncertain when based solely on morphology.

Among the above studies, Davis (2004), He-Nygrén et al. (2004, 2006), Heinrichs et al. (2005), Forrest et al. (2006), and Hentschel et al. (2006) sampled *Neotrichocolea*. All molecular analyses indicated that *Neotrichocolea* was closely related to *Ptilidium*; however, the position of this *Ptilidium–Neotrichocolea* clade within the leafy liverworts was ambiguous. In these molecular studies, leafy liverworts are divided into two major clades, which were named "Leafy I" and "Leafy II" by Davis (2004). The *Ptilidium–Neotrichocolea* clade has been resolved in "Leafy I" or "Leafy II" by different studies, but no results have received strong support. Until now, *Trichocoleopsis* has not been included in any molecular study.

In this study, we generated sequence data from four chloroplast genes (small subunit [SSU], large subunit [LSU], *atpB*, and *rbcL*) for *Trichocoleopsis* and *Neotrichocolea*, and two nuclear ribosomal (18S and 26S), one mitochondrial (*nad5*), and two chloroplast (*psbA* and *rps4*) genes for *Trichocoleopsis*. Two different data sets were constructed with different taxon and character sampling schemes. Using these data, we aim to: (1) determine the position of *Trichocoleopsis* within the leafy liverworts, and (2) clarify the relationship between *Trichocoleopsis* and *Neotrichocolea*.

Materials and Methods

TAXON SAMPLING

The majority of taxa sampled for this study were derived from the recent four-gene analysis reported by Davis (2004) and the six-gene analysis by Qiu et al. (2006). *Trichocoleopsis sacculata* was collected by Liu Yang (collection number *4170*) in 2005 from Mt. Huang, Anhui Province, China (Table 1), and *Neotrichocolea bisseti* by He Lin (collection number *040522*) in 2004 from Mt. Fanjing, Guizhou Province, China (Table 1). The voucher specimens have been deposited in the herbarium of the Institute of Botany, the Chinese Academy of Sciences, Beijing (PE). This study obtained four new sequences for *N. bisseti*: chloroplast SSU ribosomal DNA (rDNA) (GenBank accession number EF460698), LSU rDNA (EF460696), *atpB* (EF460694), and *rbcL* (EF460702); and nine new sequences for *T. sacculata*: chloroplast SSU rDNA (EF460697), LSU rDNA (EF460695), *atpB* (EF460693), *rbcL* (EF460701), *rps4* (EF460703), and *psbA* (EF460700), nuclear SSU (18S) rDNA (EF460691) and LSU (26S) rDNA (EF460692), and mitochondrial *nad5* (EF460699). For the four-gene data set, 57 taxa representing 57 genera in 34 families of liverworts were included. The sampling came mostly from Davis (2004) and focused on the leafy liverworts. For the nine-gene data set, including 24 genera of 19 families, the strategy was to further stabilize the tree topology as sequence data increased. Although taxon density of this data set was lower than that of the four-gene data set, all major lineages of leafy liverworts were sampled. According to previous studies (Davis, 2004; He-Nygrén et al., 2004, 2006; Heinrichs et al., 2005; Forrest et al., 2006), six genera from simple thalloid liverworts were chosen as outgroups in all analyses. In this study, classification followed Crandall-Stotler and Stotler (2000).

MOLECULAR AND PHYLOGENETIC ANALYSES

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing protocols followed Davis (2004) and Liu et al. (2005). All alignments were made using CLUSTAL X 1.83 (Thompson et al., 1997) and then adjusted manually in BioEdit 5.0.9 (Hall, 1999). By comparing the alignments made by Davis (2004) and our own, ambiguous positions (mainly in the *nad5* and 26S) were excluded from phylogenetic analyses. Because we were unable to sequence the 18S gene from *Neotrichocolea*, despite repeated attempts, this sequence was coded as missing data. The resulting matrices have been deposited in TreeBASE (<http://www.treebase.org>; accession number SN3273).

Phylogenetic analyses for each matrix were carried out using maximum parsimony (MP) and maximum likelihood (ML) methods in PAUP* version 4.0b10 (Swofford, 2003) and PHYML version 2.4.3 (Guindon & Gascuel, 2003), respectively. For MP analyses, heuristic searches were conducted with 1000 replicates of each 100 random sequence addition, tree bisection-reconnection (TBR) branch swapping, MulTrees in effect. All characters were unordered and equally weighted, and gaps were coded as missing data. To assess node support, bootstrap analyses (Felsenstein, 1985) were performed using 1000 heuristic search replicates as described above. Likelihood analysis was performed using a general time reversible (GTR) substitution model with invariant sites and additional among-site rate variation

Table 2. Comparison of 57-taxon and 24-taxon MP tree statistics. CI, consistency index; RI, retention index.

	Total characters	Variable characters	Variable informative sites (%)	Trees	Tree length	CI	RI
57 Taxa							
Nuclear only	1077	244	130 (12.1%)	510	686	0.484	0.650
Mitochondrial only	1125	479	241 (21.4%)	12,360	946	0.685	0.687
Chloroplast only	1764	819	660 (37.4%)	4	3756	0.319	0.521
4-Gene combined	3966	1497	1028 (25.9%)	4	5356	0.386	0.549
24 Taxa							
Nuclear only	2391	356	132 (5.5%)	9	672	0.641	0.516
Mitochondrial only	1052	318	130 (12.4%)	14,445	499	0.741	0.584
Chloroplast only	6619	1891	1277 (19.3%)	1	5359	0.477	0.422
9-Gene combined	10,062	2565	1539 (15.3%)	3	6586	0.509	0.430

modeled as a discrete gamma distribution (Yang, 1994). ML parameter values were then optimized, with a BIONJ tree as a starting point (Gascuel, 1997) with the appropriate parameters. Nodal robustness on the ML tree was estimated by the nonparametric bootstrap (1000 replicates).

RESULTS

FOUR-GENE ANALYSIS

This data set included nuclear 26S (1077 sites), chloroplast *psbA* (1171 sites) and *rps4* (593 sites), and mitochondrial *nad5* (1125 sites) genes for 57 taxa. Of the total 1028 informative characters, 64.2% and 23.4% derived from the chloroplast and the mitochondrial gene partitions, respectively. MP analysis resulted in four maximally parsimonious trees (MPTs) of length 5356. The consistency index (CI) and retention index (RI) values are consistently higher for mitochondrial genes than for the nuclear or chloroplast genes (Table 2). Topologies resolved by MP and ML analyses are mostly congruent (Fig. 2).

Leafy liverworts are recognized as monophyletic with strong support (97%$^{\mathrm{ML}}$; 92%$^{\mathrm{MP}}$). ML analyses subdivided leafy liverworts into two major clades, "Leafy I" (70%) and "Leafy II" (76%), but the "Leafy I" clade is not found in the strict consensus of four MPTs. In all analyses, *Trichocoleopsis* and *Neotrichocolea* formed a clade (71%$^{\mathrm{ML}}$; 63%$^{\mathrm{MP}}$) sister to *Ptilidium* with strong support. The *Ptilidium*–(*Trichocoleopsis*–*Neotrichocolea*) clade was resolved in "Leafy II" and sister to the remaining taxa (76%$^{\mathrm{ML}}$; 70%$^{\mathrm{MP}}$).

NINE-GENE ANALYSIS

This data set contained nuclear 26S (1061 sites) and 18S (1330 sites); chloroplast SSU (892 sites), LSU (2162 sites), *atpB* (972 sites), *rbcL* (835 sites), *rps4* (590 sites), and *psbA* (1168 sites); and mitochondrial *nad5* (1052 sites) genes for 24 taxa. Of the total 1539 informative characters, 83.0% and 8.4% derive from the chloroplast and the mitochondrial gene partitions, respectively (Table 2). MP analysis recovered three MPTs of length 6586. The ML tree was largely congruent with the MPTs (Fig. 3). Although taxon sampling was more limited compared with the four-gene data set, the results were similar to the four-gene analysis. *Trichocoleopsis* and *Neotrichocolea* formed a clade (81%$^{\mathrm{ML}}$; 84%$^{\mathrm{MP}}$), sister to *Ptilidium* with strong support, but the *Ptilidium*–(*Trichocoleopsis*–*Neotrichocolea*) clade was identified as sister to "Leafy I" in ML analysis without significant support (50%). The *Ptilidium*–(*Trichocoleopsis*–*Neotrichocolea*) clade was again resolved in "Leafy II" by MP analysis, also without significant support (55%).

DISCUSSION

TRICHOCOLEOPSIS AND *NEOTRICHOCOLEA* ARE NOT MEMBERS OF TRICHOCOLEACEAE AND LEPIDOLAENACEAE

Some authors (e.g., Nakai, 1943; Müller, 1951–1958; Schuster, 1957; Mizutani & Hattori, 1969; Mizutani, 1972) placed *Trichocoleopsis* and/or *Neotrichocolea* in Trichocoleaceae, based mainly on the presence of a coelocaule. However, as pointed out by Schuster (1966, 1972), there are numerous characters suggesting the remoteness of the relationship between them (Table 3). For example, the former two genera have a coelocaule with simple paraphyllia (usually branched in Trichocoleaceae), incubous leaves (succubous in Trichocoleaceae), relatively reduced underleaves (similar to the lateral leaves in Trichocoleaceae), freely developed rhizoids (usually lacking in Trichocoleaceae), water sacs (absent in Trichocoleaceae), unelongated leaf cells, collenchyma, smooth cilia cells (striolate-papillose in Trichocoleaceae), and relatively large spores (40–55 μm in *Trichocoleopsis* and *Neotrichocolea* but 13–20 μm in Trichocoleaceae). In fact, Asakawa et al. (1981b) hypothesized that *Trichocoleopsis* and *Neotrichocolea* had a distant

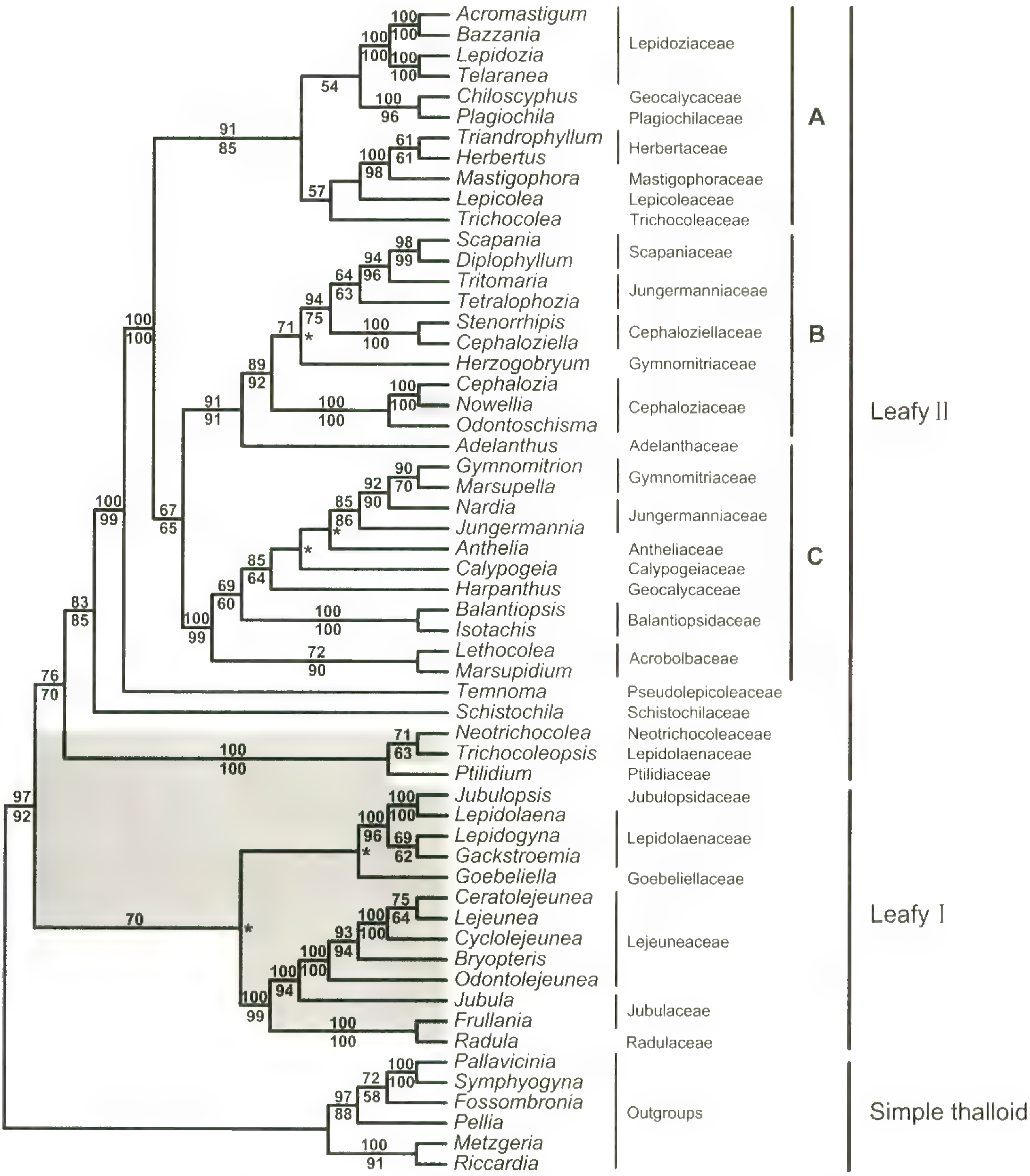

Figure 2. ML tree inferred from the four-gene data set. Numbers above and below branches are ML and MP bootstrap percentages (≥ 50%). Asterisks indicate the nodes not found in the strict consensus of MPTs. Shading represents those genera having leaf water sacs. Higher family names follow the classification of Crandall-Stotler and Stotler (2000). The circumscription of the Leafy I and II liverworts and clades A, B, and C beside the tree are based on Davis (2004).

relationship with *Trichocolea* Dumort., because their chemical components are quite different. In the present molecular analyses, Trichocoleaceae was resolved as a member of the strongly supported clade A in "Leafy II," congruent with previous studies (e.g., Davis, 2004; He-Nygrén et al., 2004, 2006; Heinrichs et al., 2005; Forrest et al., 2006). It is clear that the two endemic genera and Trichocoleaceae belong to two remote clades, respectively (Figs. 2, 3).

In modern classification of liverworts, the placement of *Trichocoleopsis* and *Neotrichocolea* in Lepidolaenaceae was also widely accepted (Schuster, 1972, 1979; Grolle, 1983; Furuki & Mizutani, 1994; Cao et al. 2006; Yamada & Iwatsuki, 2006). However, in this study, Lepidolaenaceae, together with Jubulopsidaceae, formed a well-supported clade ($100\%^{ML}$; $96\%^{MP}$) (Fig. 2), which was consistent with the results of previous molecular studies (Davis, 2004; He-

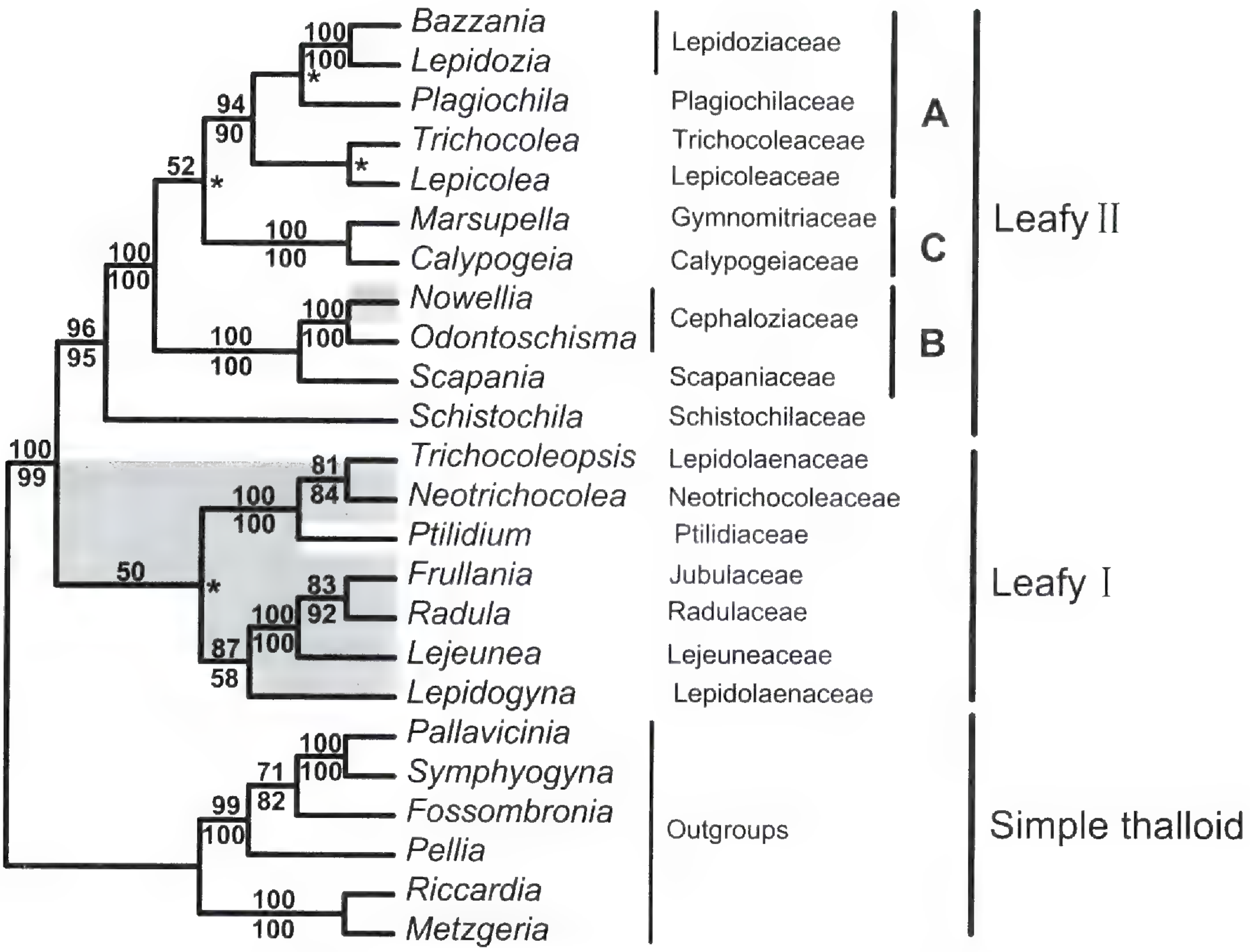

Figure 3. ML tree inferred from the nine-gene data set. Numbers above and below branches are ML and MP bootstrap percentages (≥ 50%). Asterisks indicate the nodes not found in the strict consensus of MPTs. Shading represents those genera having leaf water sacs. Higher family names follow the classification of Crandall-Stotler and Stotler (2000). The circumscription of the Leafy I and II liverworts and clades A, B, and C beside the tree are based on Davis (2004).

Nygrén et al., 2004, 2006; Heinrichs et al., 2005). In the aligned matrix of the *nad5* sequences, there are several unique insertions ("GCACGA" insertion at 740–745, "CAC" at 992–994, and "AAGA" or "AATA" at 1010–1013) shared by Lepidolaenaceae and Jubulopsidaceae, but they are not found in *Trichocoleopsis*, *Neotrichocolea*, or other taxa, which provides additional evidence for the distant relationship between them.

Initially, *Trichocoleopsis* and *Neotrichocolea* were placed in Lepidolaenaceae (Schuster 1966, 1979), based mainly on the endosporic development of the protonema and bearing water sacs. However, there are remarkable differences between the two genera and Lepidolaenaceae. In Lepidolaenaceae, the interior cells of the capsule wall have I- or U-shaped secondary thickenings (Grolle, 1967), but they are extremely thin walled in *Trichocoleopsis* and *Neotrichocolea*. Additionally, water sacs occur both on lateral leaves and underleaves in Lepidolaenaceae, but only on lateral leaves in *Trichocoleopsis* and *Neotrichocolea*. In Lepidolaenaceae, the water sac is derived from the middle lobe of the trifid leaf; by contrast, this structure originates from the ventral-most lobe in *Trichocoleopsis* and *Neotrichocolea*. Additionally, Lepidolaenaceae is distributed in Gondwanian regions, whereas *Trichocoleopsis* and *Neotrichocolea* are endemic to eastern Asia. If the two genera were considered members of Lepidolaenaceae, this disjunction would be so rare that Schuster (1983), under this assumption, once called *Trichocoleopsis* and *Neotrichocolea* "the most striking hepatic endemics in eastern Asia."

THE RELATIONSHIP BETWEEN *TRICHOCOLEOPSIS* AND *NEOTRICHOCOLEA*

Recent molecular studies all support that *Neotrichocolea* is closely related to *Ptilidium* (Davis, 2004; He-Nygrén et al., 2004, 2006; Heinrichs et al., 2005; Forrest et al., 2006; Hentschel et al., 2006). Our results showed that *Neotrichocolea* had a closer relationship with *Trichocoleopsis* than with *Ptilidium*, although the three genera formed a strongly supported monophyletic group (Figs. 2, 3). Inoue (1974) indicated that *Trichocoleopsis* and *Neotrichocolea* were

Table 3. Comparison of morphological characters in *Trichocoleopsis*, *Neotrichocolea*, and other related genera as described in detail by Schuster (1966, 1972), Grolle (1967), and Inoue (1974). NA, not available.

Character	*Trichocoleopsis*	*Neotrichocolea*	*Ptilidium*	*Lepidolaena* Dumort.	*Trichocolea*
Branching types	1 to 2 pinnately branched, *Frullania* type	3 to 4 pinnately branched, *Frullania* type	1 to 3 pinnately branched, *Frullania* type	2 to 4 pinnately branched, *Frullania* type	1 to 3 pinnately branched, *Frullania* type
Subfloral innovation	present	present	absent	present	present
Leaf insertion pattern	incubous	incubous	transverse (upper end incubous)	incubous	succubous
Leaf and underleaf	lobed, margins ciliate, cilia cells smooth, underleaf smaller than lateral leaf	lobed, both margin and dorsal surface of leaf ciliate, cilia cells smooth, underleaf smaller than lateral leaf	lobed, margins ciliate, cilia cells smooth, underleaf smaller than lateral leaf	not lobed, margins without or with short cilia, cilia cells smooth, underleaf smaller than lateral leaf	strongly lobed, margins ciliate, cilia cells cuticle striolate-papillose, underleaf similar to lateral leaf
Water sacs	present	present	absent	present	absent
Leaf cells and oil bodies	irregularly polygonal, with small trigones, oil bodies granular botryoidal	round rectangular, with weak trigones, oil bodies granular botryoidal	irregularly polygonal, with bulging trigones, oil bodies nearly homogeneous	irregularly polygonal, with bulging trigones, oil bodies nearly homogeneous	rectangular, without obvious trigones, oil bodies nearly homogeneous
Rhizoids	present	present	absent	NA	absent
Perianth	absent, having coelocaules	absent, having coelocaules	present	absent, having coelocaules	absent, having coelocaules
Capsule	apex rounded, epidermal cells with secondary thickenings at corners, interior cells thin walled, easily removed	apex rounded, epidermal cells with secondary thickenings at corners, inner cells thin walled, easily removed	apex beaked, epidermal cells with nodular thickenings, inner cells thinner with semicircular thickenings	apex beaked, epidermal and inner cells equally sized, all have I- or U-shaped secondary thickenings	apex rounded, epidermal cells hyaline, without secondary thickenings, inner cells with distinct secondary thickenings
Spore diameter	48–55 µm	40–48 µm	25–27 µm	50–80 µm	13–20 µm
Sporeling type	endosporous, *Trichocoleopsis* type	endosporous	exosporous, *Nardia* type	endosporous, *Frullania* type	exosporous, *Nardia* type

closely allied to each other. Indeed, the two genera are similar in many morphological characters, such as the lack of a perianth, the presence of a paraphyllose, massive coelocaule, and the interior cells of the capsule wall being extremely thin walled and easily removed. Additionally, they all possess incubous leaves, with cilia on the lobed leaf margins, water sacs, and semblable oil bodies (Table 3). Moreover, a similar distribution pattern (Fig. 1) and habitat (Chen & Wu, 1965) are shared by the two genera. However, as described by Hattori (1947), there are enough differences to separate them at the generic level: cilia occur only on leaf margins in *Trichocoleopsis* but both on the margin and dorsal surface of leaves in *Neotrichocolea*; and water sacs are present on stem and branch leaves of *Trichocoleopsis* but only on branch leaves in *Neotrichocolea*. Moreover, the development and shape of water sacs are distinct in the two genera.

Water sacs are widely observed in certain members of "Leafy I" (see shading in Figs. 2, 3) and were regarded as a device for facilitating water storage (Schuster, 1984). Their shapes can be cup-like (in *Frullania* Raddi), horn-shaped (in *Goebeliella* Steph.), or even specialized by having a trap-like structure in *Colura* (Dumort.) Dumort. and *Pleurozia* Dumort., which are considered to have a zoophagy function (Hess et al., 2005). According to development in dissection, saccate lobules are divided into two kinds: the "*Lejeunea* Lib. type" and "*Frullania* type." A *Lejeunea*-type water sac comes from an inrolling of the free margin of the lobule against the back side of the lobe and is formed from both lobe and lobule tissue. A *Frullania*-type water sac, in contrast, involves the development of a secondary meristematic region in the midzone of the lobule. At an early stage, the lobule inrolls to the dorsal lobe. Afterward, continued divisions cause the upper part of the lobule to balloon or inflate down upon itself (Crandall-Stotler & Guerke, 1980). The opening of this kind of lobule faces away from the apex of the stem.

Trichocoleopsis and *Neotrichocolea* were described as having *Lejeunea*-type (Shuster, 1972) and *Frullania*-type (Evans, 1905) water sacs, respectively. In *Trichocoleopsis*, the free margin of the lobule is deeply inrolled, and it can be drawn open like a spring. This type of deep inrolling is also found in many Lejeuneaceae (such as *Neurolejeunea* (Spruce) Schiffn., *Mastigolejeunea* (Spruce) Schiffn., and *Siphonolejeunea* Herzog). As in other *Lejeunea*-type lobules, a *Trichocoleopsis* lobule is inserted in a U line on the stem, so the whole leaf has a J-shaped insertion. In *Neotrichocolea*, the *Frullania*-type water sac is a slipper-like structure, opening parallel to the stem, but is not cup-like as in *Frullania*. It is remarkable that genera with such different lobule development patterns are so closely related. *Trichocoleopsis* and *Neotrichocolea* do share unique lobule position: water sacs of both come from the ventral-most lobe of the trifid leaf. In contrast, other *Lejeunea*-type and *Frullania*-type water sacs are typically derived from the middle lobe of the trifid leaf. The third lobe can convert to a stylus, as in certain species of *Frullania* and Lepidolaenaceae, or be reduced to a slime papilla as in Lejeuneaceae and *Jubula* Dumort. (Schuster, 1972).

AFFINITIES OF *TRICHOCOLEOPSIS* AND *NEOTRICHOCOLEA*

In the present study, analyses of the four- and nine-gene data sets all strongly support *Ptilidium* as sister to *Trichocoleopsis* and *Neotrichocolea*. In the alignment of *nad5* sequences, there was an exclusive "CAT" insertion at the position of 593–595 shared by *Neotrichocolea*, *Trichocoleopsis*, and *Ptilidium*, which further supported the close relationship between them. *Trichocoleopsis* was described as having a special endosporic sporeling type (*Trichocoleopsis* type [Inoue, 1956]), which is different from the exosporous *Nardia* Gray type of *Ptilidium*. However, Inoue (1963) described that in *T. sacculata*, the early stage of development of the leafy shoot was essentially the same as that of *Ptilidium*, indicating a potential relationship between them. Additionally, a unique furanosesquiterpene, deoxopinguisone, and its derivatives were shared by *Ptilidium* and *Trichocoleopsis* (Asakawa et al., 1981a).

Ptilidiaceae, once a portmanteau family of liverworts, was suggested to contain 15 genera by Evans (1939). Over subsequent years, most of these were gradually removed from the family, until only *Ptilidium* remained (Schuster, 1979, 1984; Grolle, 1983; Crandall-Stotler & Stotler, 2000). *Trichocoleopsis* and *Neotrichocolea* were previously placed in Ptilidiaceae (Evans, 1939; Hattori, 1947; Schuster, 1966; Mizutani & Hattori, 1969). Some key differences in morphology between the *Trichocoleopsis*–*Neotrichocolea* group and Ptilidiaceae precipitated their removal (Table 3). For example, *Ptilidium* has a well-developed perianth, which was considered the most important difference between *Ptilidium* and the *Trichocoleopsis*–*Neotrichocolea* group, and also between Trichocoleaceae and Lepidolaenaceae (Schuster, 1972; Inoue, 1974). Additionally, *Trichocoleopsis* and *Neotrichocolea* have water sacs on their leaves (absent in *Ptilidium*), subfloral innovations and rhizoids (absent in *Ptilidium*), rounded capsules (beaked in *Ptilidium*), much larger spores, and different oil body types than *Ptilidium* (Table 3).

Phytogeographically, *Trichocoleopsis* and *Neotrichocolea* are endemic to East Asia, but *Ptilidium* has a holarctic distribution pattern. Schuster (1983) presumed that *Trichocoleopsis*, *Neotrichocolea*, and some other eastern Asian endemic liverwort genera, such as *Nipponolejeunea* Hatt., *Scaphophyllum* Inoue, and *Hattoria* R. M. Schust., might have had a wider range before and formed their modern distribution patterns because of extinctions in other regions. For example, the two-species genus *Nipponolejeunea* is endemic to northeastern Asia, but another amber fossil species from the Oligocene was found in Europe (Grolle, 1981). Such endemism seemed to be a relict. Thus, excessive extinctions of taxa between *Ptilidium* and the *Trichocoleopsis–Neotrichocolea* group might have occurred, which could explain the morphological gap between them. Indeed, *Trichocoleopsis* and *Neotrichocolea* may retain many primitive characters compared to *Ptilidium*, such as possessing water sacs and endosporous protonemata.

POSITION OF THE *PTILIDIUM–(TRICHOCOLEOPSIS–NEOTRICHOCOLEA)* CLADE

Based on different sampling density (taxon and gene) and analysis methods, previous molecular studies (e.g., Davis, 2004; He-Nygrén et al., 2004, 2006; Heinrichs et al., 2005; Forrest et al., 2006) have identified *Ptilidium* and/or *Neotrichocolea* close to "Leafy I" or "Leafy II," or as a part of basal leafy polytomy. However, none of these studies obtained support for these placements. Recently, Hendry et al. (2007) reported that the New Zealand endemic genus *Herzogianthus* R. M. Schust. seemed to be related to the *Ptilidium–Neotrichocolea* clade, but the position of this clade and the endemic genus within liverworts remained unresolved. According to our analyses, the *Ptilidium*–(*Trichocoleopsis–Neotrichocolea*) clade was resolved with moderate support as a member of "Leafy II" based on the ML and MP analyses of the four-gene data matrix, and was placed together with "Leafy I" in the ML analyses of the nine-gene matrix without significant support. The addition of the genus *Trichocoleopsis* did not provide enough new information to definitively resolve the position of the *Ptilidium*–(*Trichocoleopsis–Neotrichocolea*) clade.

This problem, and its implications for understanding morphological evolution in the leafy liverworts, have been discussed in detail in recent studies (see Forrest et al., 2006). Both Heinrichs et al. (2005) and He-Nygrén et al. (2006) suggested that "Leafy I" and the "*Ptilidium* clade" should be grouped in the order Porellales, even though this relationship was not supported in their molecular analyses. Various shared morphological characters were cited to support this viewpoint, such as the mutual *Frullania* branching type, lack of ventral branching, incubous leaves, endogenous spore germination (however, *Ptilidium* lacks this trait), and some chemical components.

CONCLUSIONS

Molecular and morphological evidence showed that *Trichocoleopsis* and *Neotrichocolea* represent a monophyletic group. The family Neotrichocoleaceae, which includes the two genera as suggested by Inoue (1974), should be recognized. The family is characterized by having incubous leaves with cilia on the lobed leaf margins, water sacs, a paraphyllose coelocaule, and extremely thin-walled interior capsule cells. Molecular results indicated that Neotrichocoleaceae was allied to Ptilidiaceae, but except for the similar ciliate and lobed leaves, there are almost no known synapomorphies shared by the two families. On the contrary, Neotrichocoleaceae differ from Ptilidiaceae in many characters. Thus, merging the three genera into one family is premature based on the current evidence. The exact position of the Neotrichocoleaceae–Ptilidiaceae clade remains unresolved in the present study. A better sampling of both sequence data and taxa is required before the problem can be resolved.

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